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Behaviour of ceria nanoparticles in standardized test media – influence on the results of ecotoxicological tests

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Abstract. The main objectives of this work were to establish the behaviour of a ceria nanopowder in different ecotoxicological media commonly used in standardized aquatic ecotoxicity tests and consequently to assess the acute and chronic ecotoxicity in two micro-invertebrates: *Daphnia magna* and *Ceriodaphnia dubia* and in a freshwater green algae: *Pseudokirchneriella subcapitata*. Different approaches to disperse the ceria nanoparticles *(i.e.* stirring, use of probe sonication, addition of humic acids) were tested and the influence on the biological endpoints was investigated. Despite the agglomeration phenomena observed in all the tested media, the results obtained indicated higher stability in the lower ionic strength media with addition of humic acid (2 $mg.L^{-1}$, TOC). No acute toxicity were observed with *D. magna*, whatever the dispersal method performed and the *n*CeO_2 concentration tested (up to 1000 $mg.L^{-1}$), as no acute toxicity was recorded with *C. dubia* following exposure to the stirring suspensions. On contrary, acute toxicity was recorded in *C. dubia* with EC_{50} values comprise between 11.9 and 25.3 $mg.L^{-1}$ using the probe sonicated suspension with or without humic acids addition. Significant chronic effect on the reproduction capability was also recorded in *C. dubia.* The estimated EC_{10} values were comprised between 2.1 and 2.9 $mg.L^{-1}$. Focusing on *P. subcapitata*, despite the different agglomerate size recorded in the tested media at the end of the exposure periods, results obtained were similar. Adverse effect on algal growth around 5 $mg.L^{-1}$ were reported (mean $EC_{10} = 4 \pm 1.8$ $mg.L^{-1}$). Those results suggested the needed for standardized testing protocol concerning the aqueous media used or the sample preparation for laboratory testing.

1. Introduction

Nanomaterials have nowadays widespread applications in industries, medicine, agricultural and environmental sector [1,2]. As a consequence, new nanoparticulate forms of chemicals, of which behavior and toxicological effects are still unknown, are expected to be introduced into air, soil and water, and imply uncertainties regarding the possible environmental impacts.

Due to their specific properties, experts agree with the fact that the environmental effects of nanoparticles cannot be predicted from the known ecotoxicity of the "classical" macroscopic material and thus, specific hazard assessment of nanomaterials or nanoparticles are needed. In a regulatory point of view, if conventional ecotoxicity tests have already shown to be useful in evaluating the hazard of nanoparticles [3], questions rise concerning the relevance of the existing methods, especially when considering the properties of the tested media and sample preparation for conducting standard laboratory testing. Focusing on the conventional aquatic toxicity bioassays, some standardized test

methods leave the choice of aqueous media with different physico-chemical properties. In view of nano-ecotoxicology, this can be seen as a drawback of the standard test protocols, since variability in nanomaterial properties, especially agglomeration phenomena, has been shown to depend on pH of the media, ionic strength and concentration of dissolved organic matter [4-6]. Moreover, debate about the preparation of nanoparticle suspensions has recently occurred and there is currently no broad consensus on the best approaches for preparing suspensions of nanomaterials in order to assess their toxicity towards aquatic organisms [7,8]. Suspension methods have involved use of strong solvent, bath or probe sonication, magnetic stirring for a broad range of time [7,9]. The use of different approaches prepare stock suspension for testing their toxicity towards aquatic organisms may lead to different suspension properties as different agglomeration state of the nanoparticles. It consequently may lead to different toxicity [10-12].

As many other nanomaterials, the use of ceria nanoparticles in number of applications is increasing rapidly and to date, this product can be found in different applications such as sun creams, outdoor paints or used as fuels catalyst [13]. As a result, an increase of direct and indirect release to water compartment is expected. For such reason, ceria nanoparticle is one of the NPs selected for priority testing by the Organization for Economic Cooperation and Development [14]. Nevertheless, the possible impacts of *n*CeO_2 on aquatic ecosystems have not been thoroughly examined and available results are mainly related to *Daphnia magna* [11,15].

The purpose of this work was to determine whether the use of different ecotoxicological media and different protocols to suspend a ceria nanopowder can influence its physico-chemicals behavior and consequently, its ecotoxicological response. Thus, agglomerate size and surface charge (ζ-potential) of a ceria nanopowder were investigated in MilliQ-water (MQ) and in different ecotoxicological media commonly used in standardized protocols (invertebrates ISO medium and moderately hard water medium). Different protocols to disperse the ceria nanoparticles (*i.e.* magnetic stirring, use of probe sonication, addition of humic acids) were tested and their influence on the ceria nanopowder dispersion was investigated. Finally, the acute and chronic effects of ceria nanoparticles on the micro-invertebrates (*Daphnia magna* and *Ceriodaphnia dubia*) and on algae (*Pseudokirchneriella subcapitata*) were studied, and the influence of the different ways to disperse the suspension on the ecotoxicity is discussed.

2. Materials and Methods

2.1. Chemicals and ecotoxicological media

A commercially available ceria nanopowder (*n*CeO_2) was purchased from Sigma Aldrich (Schnelldorf, Germany). The primary *n*CeO_2 particle diameter as provided by the manufacturer was below 25 nm. A same batch was used for the all experiments.

The humic acids (HA) concentrated stock solution was prepared by dissolving a dry powder of humic acids sodium salt (Sigma Aldrich, Schnelldorf, Germany) in MilliQ-water and stirring vigorously for 30 minutes. Undissolved particles were removed by filtration through a 0.2 µm membrane. The stock solution of HA was kept at 4 ± 2°C, in the dark.

The micro-invertebrates ISO and Moderately Hard Water (MHW) media were prepared as described in the following standardized protocols: OECD 202 test guideline [16] and ISO 20665 standard [17]. The OECD algae growth medium was prepared following the OECD 201 test guideline [18]. Main components and physico-chemical parameters of these media are summarized in table 1.

Table 1. Physicochemical parameters of investigated media.

		pH	Hardness (mg/L)	Conductivity (µS/cm)	Main macro-nutrients
Micro-invertebrates media	ISO (OECD 202)	7.6	250	650	$CaCl_2$, $MgSO_4$, $NaHCO_3$
	Moderately hard water (ISO 20665)	7.7	85	300	$NaHCO_3$, $MgSO_4$, $CaSO_4$
Algae growth medium	OECD (OECD 201)	8.1	25	165	$NaHCO_3$, $CaCl_2$, NH_4Cl, $MgSO_4$

2.2. Preparation of the nanoparticle dispersions

All the suspensions of *n*CeO_2 were prepared in glass flask by weighting and directly adding the nanopowder in the different media or in MilliQ water. Addition of HA from the stock solution to the media was performed prior to the powder addition in order to obtained a final concentration of 2 $mg.L^{-1}$, total organic carbon (TOC). The *n*CeO_2 suspensions were dispersed using a vigorous 24h magnetic stirring or probe sonication (1 minute / 70 watts). Fresh suspensions were prepared extemporaneously.

The *n*CeO_2 suspensions were characterized for particle size (Z-average) by dynamic light scattering (DLS) particle sizer and for surface charge (ζ-potential, in mV), (Zetasizer, Malvern Instruments zetasizer). Light microscopy (Axio Imager, Zeiss®) and transmission electron microscopy (CM 12, Philips®) were used to visualize the agglomeration state of *n*CeO_2 in the different suspensions. For transmission electron microscopy (TEM), carbon coated grids were hydrophilised using an Emitech K100X glow discharger (10^{-1} mbar/40 mA/3 minutes) prior to the suspension deposition, to limit artifactual agglomeration phenomenon during the grids preparation [19].

2.3. Acute and chronic ecotoxicity tests

The ecotoxicity tests were performed on the following fresh water micro-invertebrate species: *D. magna* and *C. dubia*, and on the fresh water green algae: *P. subcapitata*. Methods, duration and followed endpoints are summarized in table 2.

Table 2. Ecotoxicity test protocols.

Organisms	*D. magna*	*C. dubia*		*P. subcapitata*
Type of toxicity	Acute	Acute	Chronic	Chronic
Test methods	OECD 202	US EPA 2002.0	ISO 20665	OECD 201
Test duration	48h	48h	8d	72h
Renewal	24h	24h	6 times *	No
Endpoint	Inhibition of mobility	Inhibition of mobility	Inhibition of reproduction	Inhibition of growth
Age of the test organisms	< 24h old	< 24h old	< 24h old	exponential growth phase
Test solution volume	10 ml	15 ml	50 ml	25 ml
Nb of organisms/flask	5	5	1	-
Condition	No stirring	No stirring	No stirring	Continuous stirring

* 6 times during exposure period (Day 0, D3, D4, D5, D6 and D7).

2.4. Statistical analysis

The Log-normal model in the REGTOX software for Microsoft Excel [20] was used for the calculation of toxicity parameters (EC*x*) and their confidence intervals. Values are drawn as mean ± standard deviation in the figures. EC*x* values are reported with 95% confidence intervals.

3. Results

3.1. Size and surface charge characterization of $nCeO_2$ in ecotoxicological media

Agglomerates state and size and surface charge (ζ-potential) of each $nCeO_2$ suspension are presented in table 3 and figure 1. The suspensions prepared using the 24h-stirring protocol without HA showed agglomerates of various sizes. As expected, the higher ionic strength media were subjected to highest agglomeration phenomena compared to the lower ionic strength media. As illustrated by the figure 1 (picture a.), the 24h-stirred suspensions in ISO medium appeared to be highly polydispersed with large agglomerate size up to hundred micrometers. Agglomerates size between 2 and 5 µm were also found. Concerning the 24h-stirred MHW and OECD media without HA, large agglomerate sizes up to several micrometers were observed (figure 1, b. and c.), submicron agglomerates around 500 nm were also noted in both media. Z-average values above 2 µm were obtained by DLS for the 24-h stirred ISO and MHW media prepared without HA. However, those values must be considered with care because of the presence of very large and settling agglomerates during the measurement and the very high polydispersity of the suspensions, which make the results less accurate. Concerning OECD media, a mean value of 1588 nm was recorded.

Not surprisingly, the use of probe sonication (1 min/ 70 watts) greatly improved the dispersion of $nCeO_2$, especially in the less ionic strength media. Z-average recorded by DLS showed mean agglomerates size of 1871 nm, 1771 nm and 864 nm in the ISO, MHW and OECD media without HA. Mean agglomerates size around 250 was recorded in the MQ water.

Relating to the surface charge of $nCeO_2$, our results showed that the |ζ-potential| of particles decrease with the increase of ionic strength of the aqueous medium used for the dispersion. A |ζ-potential| from 8 to 9 mV was recorded for the highest ionic strength ISO medium prepared without HA addition, which was two-fold lower than the value recorded in the lowest ionic strength OECD medium without HA addition.

Table 3. Mean diameter ± standard deviation (Z-average in nm) and mean value of the ζ-potential ± standard deviation (in mV) of ceria in the different ecotoxicological media and in MilliQ water using a 24h-stirring or a 1 minute-probe sonication as dispersion method. All values are reported as mean (± standard deviation) of 10 different preparations (3 measurements per preparation), except for ISO media (only one preparation).

	Aqueous media	Mean particles size (Z-average, nm)	Pdi*	|ζ-potential| mV (± S.D)	pH
24h-stirring	ISO	*2346* [(a)]	>1	9	7.3
	ISO + AH	*1736* [(a)]	>1	16	7.1
	MHW	*3180 (±817)* [(a)]	>1	16 (± 0.7)	7.4
	MHW + AH	931 (± 235)	0.7	19 (± 0.4)	7.4
	OECD	1588 (± 479)	0.6	18 (± 1.3)	7.7
	OECD + AH	431 (± 68)	0.5	21 (±0.5)	7.7
	MQ	863 (±482)	0.7	20 (± 6.8)	7.8
	MQ + AH	542 (± 71)	0.5	35 (± 2.6)	5.1
Probe sonication 1 minute	ISO	*1871* [(a)]	>1	8.6	n.a
	ISO + HA	1126	0.4	16	n.a
	MHW	1771.8 (± 425)	0.4	16 (± 0.7)	7.6
	MHW + HA	229 (± 15)	0.3	19 (± 0.4)	7.5
	OECD	864 (± 500)	0.3	18 (±0.4)	7.7
	OECD + HA	222 (± 15)	0.3	23 (±2.3)	7.7
	MQ	246 (± 26)	0.3	31 (± 3.3)	7.8
	MQ + HA	221 (± 4)	0.3	38 (± 2)	6.2

* Polydispersity index
n.a: not available
(a) to consider with care because sample may be not suitable for DLS measurement (presence of large and settling particles)

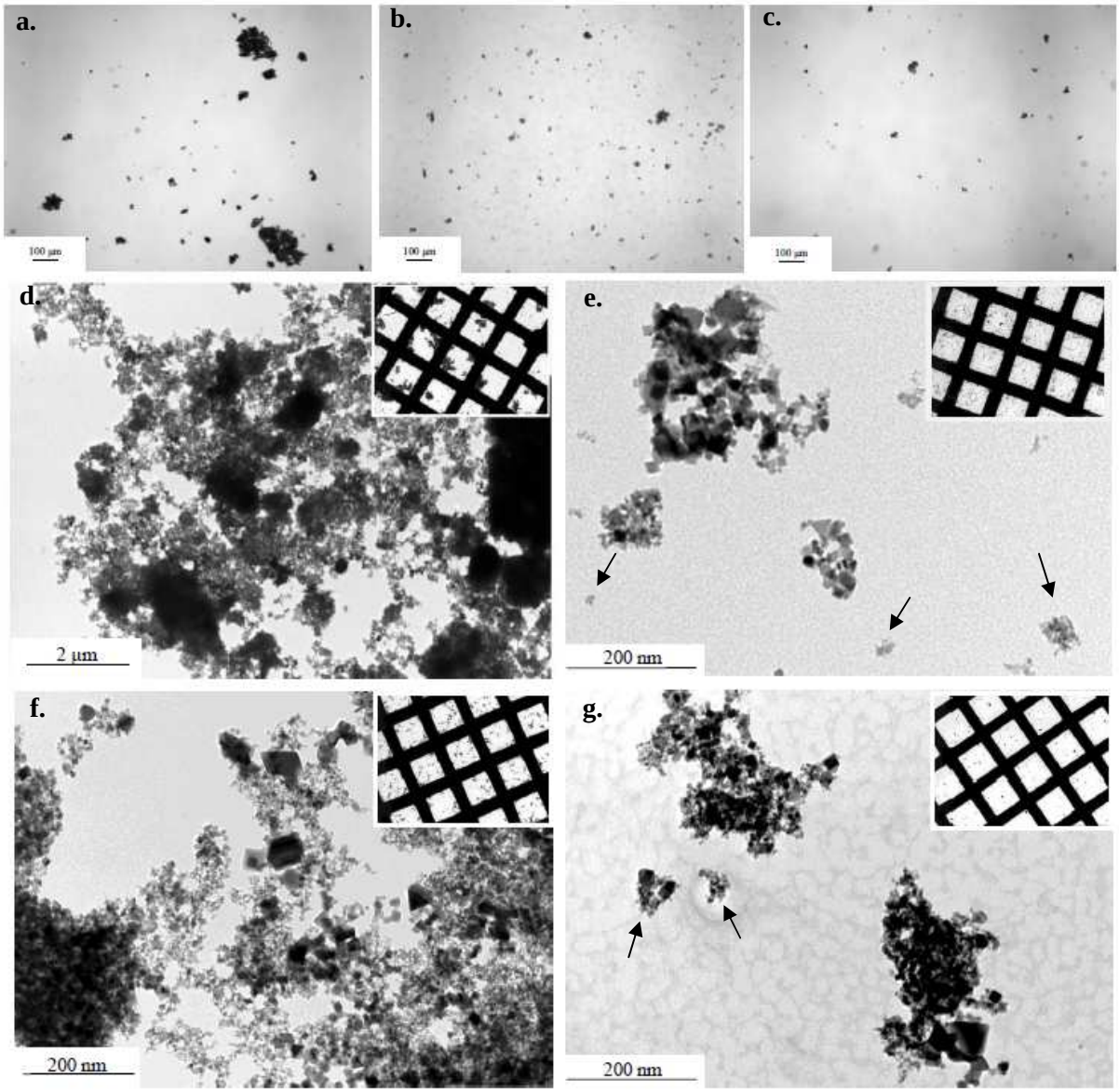

Figure 1. Light microscope images and TEM micrographs showing *n*CeO_2 agglomerates in the different ecotoxicological media, after 24h-stirring (picture a., b. and c.) or probe sonication (pictures d., e., f. and g.). After 24-h stirring, largest agglomerates are visible in the ISO medium without HA addition (picture a.). Smaller agglomerates were noted in MHW and OECD media (pictures b. and c., respectively). Pictures d. and e., show the *n*CeO_2 TEM micrographs of *n*CeO_2 suspensions dispersed using probe sonication in MHW medium without HA (picture d.) and with HA addition (picture e.). Pictures f. and g., show the *n*CeO_2 TEM micrographs of *n*CeO_2 suspensions dispersed using probe sonication in OECD medium without HA (picture f.) and with HA addition (picture g.). Black arrows highlight very small agglomerates of *n*CeO_2 below 100 nm.

Upon addition of HA (2 $mg.L^{-1}$, TOC) to the different media, a significant increase of the |ζ-potential| was recorded whatever the suspension investigated. The mean agglomerates size was also reduced whatever the media tested or the dispersion methods performed. That may be explaining by the adsorption of HA to the particles surface which enhances the particles charge and thus reduce the agglomeration phenomena. As illustrated by figure 2, it was shown that the increase of the |ζ-potential| above 20 mV has significantly reduced the agglomeration phenomena and the polydispersity of the

different suspensions. The use of probe sonication combined with HA acid addition was particularly efficient to disperse the ceria nanopowder in MHW media, OECD media as in MQ water. Using this protocol, the Z-average recorded by DLS showed mean agglomerates size from 221 to 229 nm. Those results were also confirmed by the TEM micrographs of MHW and OECD suspensions which indicate large and loose agglomerates in the absence of HA addition (figure 1, d. and f.), when smaller agglomerates are observed in the sonicated media prepared with HA (figure 1, e. and g.). As for MQ water, the sonicated suspensions in MHW and OECD media with HA addition appeared to be less polydispersed and more stable in time, with small agglomerates which fell in the field of "nanoparticles" definition (figure 1, pictures e. and g.).

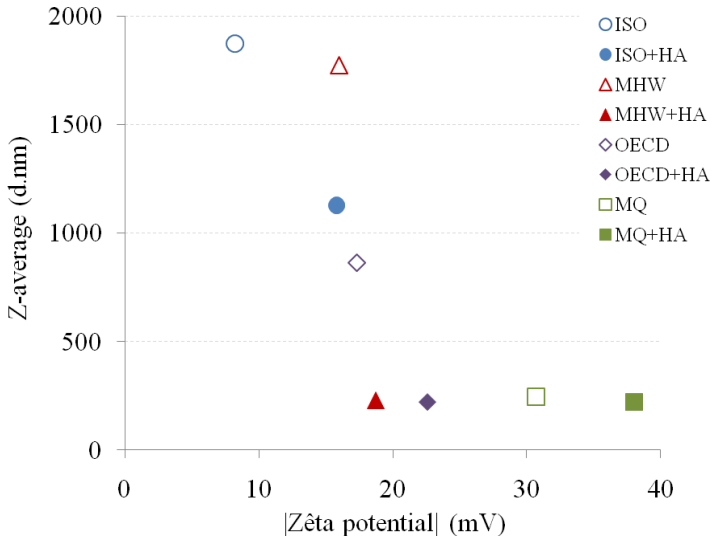

Figure 2. Physico-chemical characteristics of *n*CeO_2 in MilliQ water and in the standardized ecotoxicological media for micro-invertebrates and algae dispersed by probe sonication. All the media were prepared with or without addition of humic acids (HA ; 2 $mg.L^{-1}$, TOC). Values are drawn as mean of 10 different preparations (3 measurements per preparation) except for ISO media (only one preparation).

3.2. Acute and chronic effects of nCeO_2 on D. magna and C. dubia

Data concerning acute ecotoxicity of *n*CeO_2 to micro-invertebrates are summarized in table 4. Regarding to both *D. magna* and *C. dubia*, no inhibition of the mobility was recorded after a 48h exposure whatever the medium tested and considering the 24h-stirring condition. Likewise, after a 48h exposure period to the probe sonicated suspensions with or without HA, no significant inhibition of the mobility of *D. magna* was recorded.

Opposite, we found a significant inhibition of the mobility in *C. dubia* after a 48h exposure to the probe sonicated suspensions with or without HA addition. The estimated EC_{50} values ranged from 13.6 to 16.8 $mg.L^{-1}$, in the media prepared without HA and from 11.6 $mg.L^{-1}$ to 25 $mg.L^{-1}$, in the media prepared with HA. A control containing 2 $mg.L^{-1}$ TOC of HA without *n*CeO_2 confirmed that HA did not affect the mobility of *D. magna* and *C. dubia*.

Whatever the condition tested, binocular observation indicated that *n*CeO_2 agglomerates were clearly visible on and within the organisms (figure 3). The vast majority of *n*CeO_2 agglomerates were found to be accumulated in the gut of the organisms and also adsorbed on the shell. After a 2 hours post-exposure period in clean medium, a clearance of the *n*CeO_2 aggregates has occurred, but residual particles remain in the gut and on the shell. Same observation was made in *C. dubia* (data not shown).

Table 4. Results of the micro-invertebrates acute toxicity tests.

Method of dispersion	Organisms	Medium	Maximal concentration ($mg.L^{-1}$)	EC_{50} ($mg.L^{-1}$)
24h-stirring	*D. magna*	ISO	1000	> 1000
		ISO+HA	1000	> 1000
		MHW	100	> 100
		MHW+HA	100	> 100
	C. dubia	MHW	100	> 100
		MHW+HA	100	> 100
Probe sonication 1 minute	*D. magna*	MHW	100	> 100
		MHW+HA	100	> 100
	C. dubia	MHW	100	13.6 (11.2 - 14.6)
		MHW+HA	100	25.3 (18 - 33.1)
		MHW	100	16.8 (15 - 17.8)
		MHW+HA	100	11.9 (9.5 - 15)

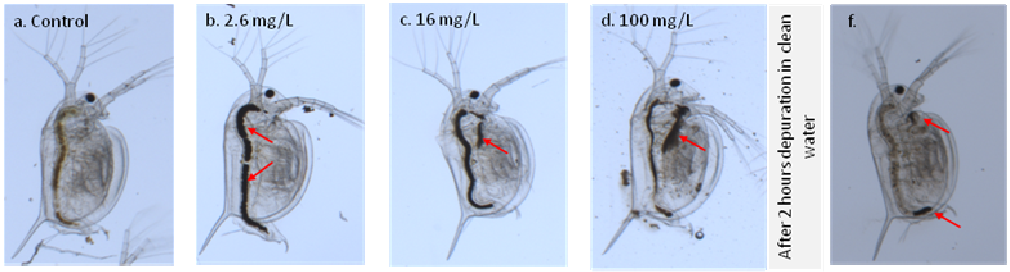

Figure 3. Binocular observation of *D. magna* after 48h exposure in nCeO$_2$ suspensions. The black color of the digestive tract shows the uptake of nCeO$_2$. Agglomerates of nCeO$_2$ were also found adsorbed on the shell. Picture f. shows *D. magna* after a 2h post-exposure period in clean medium. Red arrows highlight the accumulated nCeO$_2$.

The *C. dubia* 8d-chronic toxicity tests were performed using MHW media prepared by stirring and probe sonication without HA addition. After exposition to the 24 h-stirring suspensions, no immobility was observed during the 8 days of exposure period, when 50% immobility were recorded in the probe sonicated suspension at 100 $mg.L^{-1}$. Surviving adults were measured at the end of the exposure period, and indicated only a slight (but significant) decrease of the size of the adults exposed at the most concentrated suspension (100 $mg.L^{-1}$) prepared by probe sonication (data not shown).

However, significant adverse effects concerning *C. dubia* reproduction were observed in the tested concentration range of the magnetic-stirred suspensions. At 100 $mg.L^{-1}$ only 3.6 neonates per exposed daphnids were observed when 15.6 neonates per exposed daphnids were recorded in the control flasks. By comparison, after exposure to the probe sonicated suspension, adverse effects were observed in the 0.3 to 100 $mg.L^{-1}$ concentration range. Only 2.9 neonates per exposed daphnids were recorded at 3.05 $mg.L^{-1}$, and no neonates were observed at the highest concentrations (*i.e.* 31.25 and 100 $mg.L^{-1}$). At 9.77 $mg.L^{-1}$, only 2 neonates were recorded for all the exposed daphnids.

The EC*x* for reproduction in the magnetic-stirring and probe sonicated suspensions are given in table 5. An EC_{50} value of 15 $mg.L^{-1}$ and an EC_{10} value of 2.9 $mg.L^{-1}$ were determined after exposure to the 24 h-stirred suspensions compared to the lower estimated EC_{50} of 2.6 $mg.L^{-1}$ and EC_{10} of 2.1 $mg.L^{-1}$ calculated in the experiment with the probe sonicated suspensions.

Table 5. Results of the *C. dubia* chronic toxicity tests.

Methods of dispersion	Medium	EC_{50} ($mg.L^{-1}$)	EC_{10} ($mg.L^{-1}$)
24h-stirring	MHW	15.7 (6.6 - 35.2)	2.9 (0.44 - 15.2)
Probe sonication 1 minute	MHW	2.6 (1.1 - 2.7)	2.1 (0.8 - 2.2)

3.3. Effect of $nCeO_2$ on P.subcapitata growth

The results obtained in the *P. subcapitata* growth inhibition tests are presented in table 6. When possible, the evolution of $nCeO_2$ agglomerates during the test was followed. A re-agglomeration was observed in the suspension prepared without humic acids, leading with agglomerates around 2 µm (measured by DLS) at the end of the experiment, when no re-agglomeration was recorded in the suspension prepared with HA addition. In those suspensions with HA addition, the agglomerate sizes stay around 350 nm in the stirring suspension and around 250 nm in the probe sonicated suspension, all over the exposure period.

Furthermore, all the estimated EC*x* values indicated a similar level of toxicity, whatever the way the suspensions were prepared and consequently the size of the particles in the suspension. Mean EC_{10} values of 4.2 $mg.L^{-1}$ and 3 $mg.L^{-1}$ were recorded in the stirring and probe sonicated suspensions, respectively. Mean EC_{50} of 14.7 $mg.L^{-1}$ and 11.5 $mg.L^{-1}$ were recorded in the stirring and probe sonicated suspensions, respectively.

Table 6. Results of the *P. subcapitata* growth inhibition test.

Method of dispersion	Medium	Stock suspension concentration	initial agglomerate size	Agglomerate size at the end of the experiment	EC_{10}	EC_{50}
		($mg.L^{-1}$)	(d. nm)	(d. nm)	($mg.L^{-1}$)	($mg.L^{-1}$)
24h-stirring	OECD	1000	nd	nd	2.8 (2.1 - 3.6)	12 (10.6 - 13.4)
	OECD	1000	nd	nd	2.9 (2 - 4.2)	14.2 (11.9 - 16.3)
	OECD	125	nd	nd	2.6 (1.8 - 3.6)	16.2 (13.9 - 19.1)
	OECD	125	nd	nd	7.5 (5.6 - 9.5)	16.4 (13.8 - 17.9)
	OECD	125	nd	1562 ± 25	5.8 (4.4 - 7.8)	12.5 (11.1 - 14.3)
	OECD+HA	125	357 ± 5.8	374 ± 10	4.1 (3.4 - 5.0)	16.9 (15.5 - 18.5)
Probe sonication 1 minute	OECD	125	647 ± 65	2782 ± 143	2.8 (2.3 - 3.3)	11.1 (10.3 - 11.9)
	OECD	125	761 ± 12	2087 ± 632	3.9 (3.4 - 4.7)	9.9 (9.1 - 10.5)
	OECD+HA	125	238 ± 2.6	253 ± 5	2.3 (1.2 - 4.1)	13.7 (10.6 - 17.1)

4. Discussion

In this study, we looked forward to investigate whether the use of different ecotoxicological media and different protocols to suspend a ceria nanopowder can influence its behavior and consequently its ecotoxicity on freshwater micro-invertebrates and algae following the conventional bioassays guidelines and standards.

4.1. Ceria nanoparticles in the standardized ecotoxicological media

From our results, the use of different ecotoxicological media and different dispersion protocols had obviously led to different behaviors of ceria nanopowder. As expected, agglomeration phenomenon was linked to the media ionic strength. This is mainly explained by the fact that the stability of a nanoparticles suspension is governed by the balance of interaction between attractive forces (such as Van-der-Waals attraction), and the surface charges repulsion from the particles [21,22]. An increase in the suspension ionic strength shields the surface charges of particles and consequently reduces the effectiveness of the electrostatic repulsion, which enhance the agglomeration process [23,24].

The use of chemical solvents to improve the suspensions stability can be controversial in ecotoxicological experiments, as they can be inherently toxic [7,25]. Several authors have shown that humic substances form surface films on colloidal particles [23,26-28] which can improve the stability of nanoparticles as previously reported on iron oxide, carbon nanotubes and fullerenes [29,30]. Thus,

HA were used as a more relevant agent relative to chemical solvents. From our data, it was shown that HA enhance the surface charges of *n*CeO_2, whatever the media investigated, which subsequently increase the electrostatic repulsion between particles. As a consequence, a decrease of agglomerates size was recorded. In the media with the lower ionic strength, a |ζ-potential| around 20 mV was enough to significantly improve the stability of the small particles in suspensions. At least, the use of probe sonication as a dispersal methods and the addition of HA (2 mg.L^{-1}, TOC), were the optimal protocol to produce small and consistent particles sizes in suspensions, with a reasonable stability over the exposure period. Moreover this protocol appears to be the most reproducible methods to disperse the ceria nanopowder in the different aqueous media.

4.2. Effect on aquatic organisms

In addition to investigate the ecotoxicity of *n*CeO_2 nanoparticles, the second objective of this work was to test the influence of the suspension preparation on the ecotoxicological effect observed. The results obtained demonstrate that *n*CeO_2 was capable of causing acute and chronic toxicity in micro-invertebrates and algae; however, toxicity may differ significantly with the species tested and the dispersal method performed to suspend the nanoparticles.

Concerning micro-invertebrates acute ecotoxicity tests, it is clear from the data reported above that susceptibility to ecotoxicity of *n*CeO_2 varies among micro-invertebrate species. *C. dubia* tend to be more susceptible to *n*CeO_2 suspensions compare with *D. magna*, especially considering exposure to the probe sonicated suspensions. Therefore, no inhibition of the mobility was recorded in *D. magna*, whatever the media used (ISO or MHW medium, with or without HA addition) or the dispersal methods performed and despite the fact that *D. magna* was clearly able to ingest a large amount of *n*CeO_2. Those findings are however in line with the data obtained by Velzeboer *et al*. [15] Van Hoecke *et al*. [11] and Lee *et al*. [31], who mentioned no acute toxicity up to 100 mg.L^{-1} with the same organisms and exposure period. Opposite, the findings recorded with *C. dubia* indicated significant (and dose-related) inhibition of the mobility when organisms were exposed to the probe sonicated suspension, while no inhibition was recorded after exposure to the magnetic stirring suspensions. Those findings obtained with *C. dubia* also highlight that the preparation and the dispersal methods have impacted the testing results.

In the chronic tests on *C. dubia*, data shown that the number of neonates was the most sensitive endpoint compared to the growth of adults. In our experiment on *C. dubia*, we have recorded EC_{50} values for reproduction of 15.7 mg.L^{-1} (6.6 - 35.2) and 2.9 mg.L^{-1} (0.44 - 15.2) and EC_{10} 2.6 mg.L^{-1} (1.1 - 2.7) and 2.1 mg.L^{-1} (0.8 - 2.2) using the 24h-magnetic stirring suspension or the probe sonicated suspension, respectively. One more time, difference in toxicity relating to the dispersal methods used was observed, indicating higher toxicity after exposure to the probe sonicated suspensions. Despite of its relevance, chronic effect of *n*CeO_2 on micro-invertebrates has rarely been investigated, and no published data on chronic toxicity to *C. dubia* of *n*CeO_2 could be found in the literature for comparison. At the best of our knowledge, only one reference was published on *D. magna* [11]. These authors also reported adverse effect of *n*CeO_2 on reproduction capability with estimated EC_{50} values between 20 and 42 mg.L^{-1} and EC_{10} values between 8.8 and 20 mg.L^{-1}, according to the primary particle size. Although those values are slightly higher, they were nevertheless in line with our results. The variation observed may be explained by the highest sensitivity of *C. dubia*, the difference in sample preparation and by difference in the primary particle size.

We hypothesize that the difference between sonicated and stirring suspension in acute or chronic ecotoxicological response recorded with *C. dubia* may be explained, at least in part, by greater fragmentation of the *n*CeO_2 agglomerate within the probe sonicated suspension and consequently, a better stability of *n*CeO_2, over the exposure period. Nevertheless, *n*CeO_2 in aqueous suspensions is not a static system and is undergoing simultaneous agglomeration and sedimentation. Hence, during micro-invertebrates toxicity test, a large amount of the initial mass, of *n*CeO_2, may be lost by sedimentation in a more or less short time, depending on the media parameters and the dispersal protocol performed. This will result in a dynamic exposure scenario, making it difficult to quantify the

real exposure concentration of *n*CeO_2 in the micro-invertebrate bioassay. Furthermore, micro-invertebrates are expected not only to swim in the middle of water column, but also to crawl in the sedimented nanopowder, making exposure a more complicated question. Therefore, the real concentrations to which the organisms were exposed may be less than reported. It is obvious form the discussion above that much research is needed concerning the exposure characterization when using static or semi-static system. Further works will focused on this point in order to refine the effect concentrations.

Finally, we have demonstrated that *n*CeO_2 induced adverse effects on the growth of *P. subcapitata*. Furthermore, despite the fact that the different dispersal protocol leads with different agglomerate size of *n*CeO_2 at the end of the exposure period, the algae growth inhibition were similar (*i.e.* mean EC_{10} = 4 ± 1.8 $mg.L^{-1}$ and mean EC_{50} = 13.9 ± 2.6 $mg.L^{-1}$). Recent results reported by Van Hoecke *et al*. [11] and Rodea-Palomares *et al*. [32] using such *n*CeO_2, but different way to prepare the testing sample (*i.e.* stabilized stock suspension in MilliQ water at low pH and drop addition in tested media, with addition of MOPS (3-(*N*-morpholino) propanesulfonic acid) buffer in the algae growth medium in the case of Van Hoecke *et al*. [11]) are also in line with our results. Van Hoecke *et al*. (2009) indicated an EC_{10} of 5.4 $mg.L^{-1}$ and an EC_{50} of 19.1 $mg.L^{-1}$ in *P. subcapitata* growth inhibition test, using CeO_2 nanoparticles with primary particle size around 29 nm and recorded agglomerates in the experimental media (OECD growth algae media) around 500 nm, when Rodea-Palomares *et al*. [32] reported an EC_{50} value around 10 $mg.L^{-1}$, using CeO_2 with primary particles size of 25 nm and recorded agglomerates around 2107 nm in the OECD growth algae media. Those findings also support the fact that the protocol to suspend *n*CeO_2 has a few influence on its toxicity when focusing on the conventional algae growth inhibition test.

5. Conclusion

In conclusion, the results above confirm that the conventional aquatic bioassays could be relevant to assess the hazard of nanoparticles, in a regulatory point of view. Nevertheless, we have demonstrated that the use of different ecotoxicological media proposed by guidelines or standards as the use of different methods to disperse nanoparticles may lead to different behaviors and consequently may influence the results obtained when performing conventional test, especially when considering the micro-invertebrates bioassays. This clearly illustrates the needed for standardized testing protocol concerning the aqueous media used or the sample preparation for laboratory testing.

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